Immunomodulatory activity of *gymnema sylvestre* R.Br.

Leaves on in-vitro human neutrophils.

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Received on: 07-04-2009; Accepted on: 20-06-2009

ABSTRACT

Immune system dysfunction is responsible for various diseases like arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer and infectious diseases. In clinical immunology laboratory tests, which utilize a great many of the recently elucidated principles of immunology can be performed. The results are then used by practicing physicians in the diagnosis, treatment and prognosis of the clinical disorders. Further more, qualitative and quantitative analysis of immune response has led to better understanding of pathogenesis of many clinical disorders. These understanding in turn have stimulated further basic scientific research in immunology. The degree to which the patient becomes abnormally susceptible to infections by this microbial environment depends on the extent of immunosuppression. For that the immunomodulatory study of the leaves *Gymnema sylvestre* R.Br. (Asclepiadaceae) was carried out by in-vitro. The aqueous extract of *Gymnema sylvestre* leaves was investigated for immunomodulatory activity by assessing Neutrophil locomotion and chemotaxis test, phagocytosis of killed *Candida albicans* and Nitroblue tetrazolium test. The extract was given at dose of 25 µg/ml, 50 µg/ml and 100 µg/ml. Results of in-vitro immunomodulatory activity lead to the conclusion that the aqueous extract of *Gymnema sylvestre* showed predominantly significant activity on in-vitro human neutrophils in all parameters, which is compared to the standard.

Keywords: *Gymnema sylvestre* leaves, Immunomodulatory, Human neutrophils, *Candida albicans*.

INTRODUCTION

Large number of plants has been screened systematically for their immunomodulatory activity. The plants chosen so far have been used prophylactically or therapeutically in folk medicine and in a non-clinical context to promote immune defence (Pallabi DE, 1998). Number of steroidal saponin glycosides have been isolated and characterised from *Panax ginseng*. Experiments conducted on standardised fractions of ginsenosides revealed potent immunomodulating activity and adaptogenic activity. Boswellic acid, isolated from *Boswellia serrata*, is a pentacyclic triterpene, possesses anti-inflammatory activity. It is widely used in India for the treatment of arthritis. Boswellic acid is believed to be a potential immunomodulating agent. The suppression of the immune system is characterized by reduction in number and phagocytic function of the neutrophils and macrophages, as well as an impairment of the intracellular bactericidal capacity of these cells. This immunosuppression allows opportunistic pathogens to overwhelm the host to cause secondary infections (Rao CS, 1994). This problem can be overcome by boosting the immune system by the use of immunomodulatory drugs (Fulzele SV, 2003). Many medicinal plants are known to have immunomodulatory properties and maintain organic resistance against infection by re-establishing the body’s immune system such as *Azadirachta indica* (Nat. VD, 1987), *Terminalia chebula* (Sohni YR, 1996), *Lawsonia alba* (Kulkarni SR, 1998) etc. The phytochemical constituents like terpenoids, steroids, proteins (Pallabi DE, 1998) and tannins (Biswa S, 2002) are considered to exhibit this immunomodulatory property.

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Preparation of extract

The shade dried leaves was subjected to physical evaluation (Trease EG, 1993). The standardized coarse powder of the leaves was subjected to water extract.

Preparation of test reagents

1) Phosphate Buffer Salt Solution (PBS)

Dissolve 2.38g of disodium hydrogen phosphate, 0.19g of potassium dihydrogen phosphate and 8.0 gm of sodium chloride in sufficient water to produce 1000ml. Adjust the pH if necessary.

2) Preparation of Test Solution

Stock solution for in-vitro studies were prepared by dissolving Water extract in 0.5 ml Dimethyl sulphoxide (DMSO) and with phosphate buffer salt solution according to concentration range from 25, 50, 100 µg/ml and undiluted extract.

3) Preparation of test sample

Samples for invitro study were prepared by dissolving 20 mg of crude extract in 20 ml of Distilled water and diluted with normal saline to obtain concentration ranging from 25, 50, and 100 µg/ml.

STUDY OF IMMUNOMODULATORY ACTIVITY

Neutrophil locomotion and chemotaxis test (Wilkinson PC, 1981)

Neutrophil cell suspension was prepared in phosphate buffer saline solution (PBS) at about 10⁶ cells/ml. The lower compartment of chemotactic chamber (5 ml beaker) was filled with appropriate chemotactic reagents pre-adjusted to a pH of 7.2 e.g. chamber 1-PBS solution (control); chamber 2-Casein 1 mg/ml (standard); and chamber 3, 4, 5, 6 and 7 with different concentrations (25, 50, and 100 µg/ml) of test sample. The upper compartment (1ml syringe) was filled with neutrophil cell suspension and the wet filter (Millipore) of 3 mm pore size was fixed at the bottom of the upper compartment. The upper compartment was placed into the lower compartment and incubated at 37º C for 2 hr.

The upper compartment was removed and inverted to empty the fluid. The lower surface of the filter was fixed with 70% ethanol for 5 min and then stained with Haematoxylin dye for 5 min. The fixed samples for invitro study were prepared by dissolving 20 mg of crude extract in 20 ml of Distilled water and diluted with normal saline to obtain concentration ranging from 25, 50, and 100 µg/ml.

Phagocytosis of killed Candida albicans (Ponkshe CA, 2002)

The Candida albicans culture was incubated in Sabouraud broth overnight and then centrifuged to form a cell button at the bottom and supernatant was discarded. The cell button was washed with sterile Hank’s Balanced Salt Solution (HBSS) and centrifuged again. This was done 3-4 times. The final cell button was mixed with a mixture of sterile HBSS and human serum in proportion of 4:1. The cell suspension of concentration 1 x 10⁹ was used for the experiment.

Slide preparation

Human blood (0.2 ml) was obtained by finger prick method on a sterile glass slide and incubated at 37º C for 25 min to allow clotting. The blood clot was removed very gently and slide was drained slowly with sterile normal saline, taking care not to wash the adhered neutrophils (invisible). The slide consisting of polymorphonuclear neutrophils (PMNs) was flooded with predetermined concentration of test sample and incubated at 37º C for 15 min.

The PMNs were covered with Candida albicans suspension and incubated at 37ºC for 1 hr. The slide was drained, fixed with methanol and stained with Giemsa stain.

Phagocytosis evaluation

The mean number of Candida cells phagocytosed by PMNs on the slide was determined microscopically for 100 granulocytes using morphological criteria. This number was taken as phagocytic index (PI) and was compared with basal PI of control. This procedure was repeated for different concentrations (25, 50, 100 µg/ml and undiluted) of test samples. Immunostimulation in % was calculated by using following equation:

Stimulation (%) = PI (test) – PI (control) X 100 / PI (control)

Qualitative nitroblue tetrazolium (NBT) test (Wilkinson PC, 1981).

A suspension of leucocytes (5 x 10⁶ /ml) was prepared in 0.5 ml of PBS solution in 7 tubes. 0.1 ml PBS solution (control) and 0.1 ml of endotoxin activated plasma (standard) is added to the 1st and 2nd tube respectively and to the other 4 tubes added 0.1ml of different concentrations (25, 50,100µg/ml and undiluted) of test samples. 0.2 ml of freshly prepared 0.15% NBT solution was added to each tube and incubated at 37ºC for 20 min. Centrifuged at 400g for 3-4 min to discard the supernatant.

The cells were resuspended in the small volume of PBS solution. A thin film was made with the drop on a slide, dried, fixed by heating, counterstained with dilute carbol-fuchsin for 15 sec. The slide was washed under tap water, dried and focussed under 100x oil immersion objective. 200 neutrophils were counted for the % of NBT positive cells containing blue granules/lumps.

RESULT

Preliminary phytochemical investigation reveals the presence of tannins and saponin. Aqueous extract have showed significant activity in some of the parameters at higher concentrations. However highly significant results were obtained in all the parameters in

Table No.1. Neutrophil Locomotion and Chemotaxis

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Normal Control (A)</th>
<th>Casein (Positive Control)</th>
<th>Aqueous Extract (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>1 µg/ml</td>
<td>[1mg/ml] B*</td>
</tr>
<tr>
<td>Mean Number of Neutrophil per Field</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>110</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>115</td>
<td>102</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>118</td>
<td>101</td>
</tr>
<tr>
<td>Mean</td>
<td>1</td>
<td>112.75</td>
<td>101</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.00</td>
<td>4.57</td>
<td>0.00</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0.00</td>
<td>2.28</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*<p value <0.001, very significant. Significant difference from positive control by One Way ANOVA followed by Dunnet’s ‘t’ test, Dunnet’s ‘t’ test (Comparisons are made between Group A & B; Group A & C). p-values: *<0.01 (B); *<0.01 (C), S.D. = Standard Deviation; S.E.M. = Standard Error of Mean. 

Undiluted 3-4 3-4
100 3-4 3-4
25 3-4 4-5

Table No3. Nitroblue Tetrazolium (NBT) Qualitative Test

<table>
<thead>
<tr>
<th>Concentration in µg/ml</th>
<th>Mean Percentage of Reduced Neutrophil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A) (B) (C)</td>
</tr>
<tr>
<td>Undiluted</td>
<td>10 58** 40**</td>
</tr>
<tr>
<td>100</td>
<td>10 58** 42**</td>
</tr>
<tr>
<td>50</td>
<td>10 58** 25**</td>
</tr>
<tr>
<td>25</td>
<td>10 58** 21*</td>
</tr>
<tr>
<td>Mean</td>
<td>10 58 32.06</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.0 0.5 10.4</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0.0 0.25 5.2</td>
</tr>
</tbody>
</table>

MPN = Mean particle number of Candida albicans

DISCUSSION

In the present study, the extract of Gymnema sylvestre leaves significantly increased the phagocytic function of human neutrophils, when compared to control indicating the possible immunostimulating effect. The engulfment of microorganisms by leukocytes called phagocytosis and which is one of the main defence mechanisms of an organism (Daniel P.S: 1987). The Gymnema sylvestre leaves extract have significantly increased the neutrophil chemotactic movement as indicated by the increase in number of cells reached the lower surface of filter, there by extracts acts as chemo attractant.

The final step of phagocytosis is the intracellular killing of microorganisms by the neutrophils, which is dependent on metabolic thrust generated through the hexose monophosphate shunt activation, and activation which is also necessary for normal microbialidal activity (Daniel PS, 1994).

The extracts have significantly increased the intracellular reduction of NBT dye to formazan (deep blue compound) by the neutrophils confirming the intracellular killing property and overall metabolic integrity of phagocytosing neutrophils.

Tannins obtained from the leaves are found to possess anti-inflammatory and immunomodulatory properties. Thus it can also be concluded that immunomodulatory activity may be due to the presence of tannins in the Gymnema sylvestre.

REFERENCES


Source of support: Nil, Conflict of interest: None Declared